

REMARKS

The April 4, 2006 Official Action and the references cited therein have been carefully reviewed. In view of the amendments submitted herewith and the following remarks, favorable reconsideration and allowance of this application are respectfully requested.

At the outset, it is noted that a shortened statutory response period of three (3) months was set forth in the April 4, 2006 Official Action. Therefore, the initial due date for response was July 4, 2006. A petition for a 1 month extension of time is presented with this response, which is being filed within the one month extension period.

It is also noted that an Information Disclosure Statement under 37 C.F.R. § 1.97 was filed August 10, 2004 in this application. It is respectfully requested that in accordance with the guidelines of Section 609 of the Manual of Patenting Examining Procedure, the Examiner consider and make of record in this application the seven (7) references listed in the Form PTO-1449, which accompanied applicants' Information Disclosure Statement.

In the April 4, 2006 Official Action, the specification is objected to for not updating the status of the parent application, U.S. Patent Application No. 09/720,841. Applicants have amended the instant priority claim to indicate that the '841 application is abandoned, thereby overcoming the instant objection.

The Examiner has also objected to claim 35 under 37 CFR §1.75(c) for allegedly being of improper form because a multiple dependent claim should refer to other claims in the alternative only. Applicants respectfully disagree with the Examiner's position. Claim 35 only depends from claim 27 and recites that the compound of formula 1 is recovered. However, in an effort to eliminate any confusion, Applicants have amended claim 35 to recite the recovery of the compound produced by the process of claim 27. Accordingly, Applicants respectfully request the instant objection be withdrawn.

The Examiner has rejected claim 26 under 35 U.S.C. §112, second paragraph for alleged indefiniteness. It is the Examiner's position that the claim omits an essential step. Applicants have cancelled claim 26, thereby rendering the instant rejection moot.

Lastly, claims 26-35 have been rejected under 35 U.S.C. §102(b) as allegedly anticipated by or, in the alternative, under 35 U.S.C. §103(a) as allegedly unpatentable over WO 98/01571.

The foregoing objections and rejections constitute all of the grounds set forth in the April 4, 2006 Official Action for refusing the present application.

In accordance with the instant amendment, Applicants have amended claims 29 and 35 (as noted above) and added claims 36-38. Support for the amendment to claim 29 can be found, for example, in original claim 25. Support for new claims 36 and 37 can be found, for example, in original claims 2 and 3, respectively. Support for new claims 38 and 39 can be found throughout the specification including, for example, at page 15, lines 20-28 and at page 16, lines 4-12.

No new matter has been introduced into this application by reason of the amendment presented herewith.

In view of the present amendment and the reasons set forth in this response, Applicants respectfully submit that the objection to the specification, the objection to claim 35, the 35 U.S.C. §112, second paragraph rejection of claim 26, and the 35 U.S.C. §§102/103 rejection of claims 26-35, as set forth in the April 4, 2006 Official Action, cannot be maintained. These grounds of objection and rejection are, therefore, respectfully traversed.

**CLAIMS 27-35 ARE NOT ANTICIPATED OR RENDERED OBVIOUS BY
WO 98/01571**

Claims 27-35 have been rejected under 35 U.S.C. §102(b) as allegedly anticipated by or, in the alternative, under 35 U.S.C. §103(a) as allegedly unpatentable over WO

98/01571. It is the Examiner's position that WO 98/01571 provides a method which anticipates the instantly claimed method or contains differences which appear to be minor in nature and obvious to a skilled artisan.

Applicants respectfully disagree with the Examiner's position. In order to constitute evidence of lack of novelty under 35 U.S.C. §102, a prior art reference must identically disclose each and every element of the rejected claim. In re Bond, 15 U.S.P.Q.2d 1566 (Fed. Cir. 1990). Further, the criterion for determining obviousness under §103 is whether the prior art supplies some motivation or incentive to one of ordinary skill in the art to arrive at the invention as claimed. In re Dow Chemical Company, 5 U.S.P.Q. 2d 1929 (Fed. Cir. 1988). The teaching or suggestion supporting the desirability or the combination must be found in the prior art, not in applicant's disclosure. In re Fritch, 23 U.S.P.Q.2d 1780 (Fed. Cir. 1992). It is also a well-settled premise in patent law that "silence in a reference is not a proper substitute for adequate disclosure of facts from which a conclusion of obviousness may justifiably follow". In re Burt, 148 U.S.P.Q. 548 (CCPA 1966). Under the above standards, WO 98/01571 fails to anticipate or render obvious the invention as claimed in claims 26-35.

The methods of the invention involve culturing a transformant organism comprising a DNA gene assembly having a loading module and a plurality of extension modules. The loading module is of the form KSq-ATq-ACP, wherein KSq is a domain operative to decarboxylate a malonate substrate carried by the ACP, ATq is an acyltransferase domain operative to load selectively a malonate unit onto the ACP, ACP being an acyl carrier protein. Further, the extension modules of the polyketide synthase are not usually associated with a loading module that effects decarboxylation of a malonyl residue.

In contrast, the polyketide synthases disclosed by WO 98/01571 contain a loading module which comprises only an AT and ACP domain (see Figure 2A and page 2, lines 18-19).

Accordingly, WO 98/01571 fails to teach loading modules comprising a KSq domain, as recited in Applicants' claims. Inasmuch as WO 98/01571 fails to teach each and every element of the instantly claimed methods, Applicants submit that the rejection of claims 26-35 as allegedly anticipated by WO 98/01571 cannot be maintained.

Further, Applicants submit that as of the priority date of the instant application, a skilled artisan would not have been motivated to include a KSq domain in the loading module of a hybrid polyketide synthase. Indeed, Khosla (Chem. Rev. (1997) 97:2577-2590) states, at page 2581, that the loading domain of spiramycin contains "a ketosynthase domain whose active-site cysteine is replaced with a glutamine (and therefore is presumably **inactive**)" (emphasis added). Khosla also states that the "loading AT-ACP of the spiramycin PKS" can be replaced with the loading domain of the tylosin PKS, thereby supporting the view in the prior art that the KSq domain is inactive and superfluous. In view of the foregoing, Applicants submit that it is clear that a skilled artisan would lack the requisite motivation and expectation of success for generating a hybrid polyketide synthase with a loading module which contains a KSq domain that is believed to be inactive. Indeed, in view of this acknowledged lack of utility in the prior art, the person of ordinary skill in the art lacks the necessary impetus to make the claimed subject matter. In re Albrecht, 185 U.S.P.Q. 585 (CCPA 1975).

In view of the foregoing, Applicants respectfully submit that the instant rejection of claims 27-35 under 35 U.S.C. §§102(b) and 103(a) is untenable and request its withdrawal.

CONCLUSION

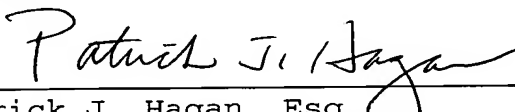
In view of the amendments presented herewith and the foregoing remarks, it is respectfully urged that the objections and rejections set forth in the April 4, 2006 Official Action be withdrawn and that this application be

passed to issue.

In the event the Examiner is not persuaded as to the allowability of any claim, and it appears that any outstanding issues may be resolved through a telephone interview, the Examiner is requested to call the undersigned agent at the phone number given below.

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Enclosure: Khosla, Chem. Rev. (1997) 97:2577-2590

Harnessing the Biosynthetic Potential of Modular Polyketide Synthases

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I. Introduction and Overview

Although the structures of polyketide natural products have been (and continue to be) elucidated for over a century, the notion that a large subset of bacterial polyketides are synthesized through the action of modular polyketide synthases (PKSs) is less than a decade old. As described below, modular PKSs are large multifunctional enzyme assemblies that catalyze the controlled biosynthesis of the carbon-chain backbones of numerous bacterial polyketides. Examples include well-known antibiotics, other pharmacologically active agents, and agricultural products [e.g., erythromycin (1), oleandomycin (2), spiramycin (3), rapamycin (4), FK506 (5), soraphen (6), avermectin (7), and candicidin (8)] (Figure 1). Indeed, the spectacular structural diversity observed among the products of modular PKSs is probably unmatched in any other family of biosynthetic products. Modular PKSs are found in both Gram-positive and Gram-negative bacteria, although they are particularly abundant in the actinomycetes.



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The discovery of modular PKSs has led to the emergence of new horizons for the engineered biosynthesis of complex natural product-like molecules. Indeed, much of the research on this subject has been motivated by the desire to develop a conceptual and technological framework for harnessing the biosynthetic potential of these remarkable protein catalysts. The goals of this review are twofold. First, I will summarize the fundamental insights into the structure and properties of modular PKSs that have been gained within the past decade. Second, currently available strategies for the use of modular PKSs to synthesize novel biomolecules will be assessed. Hopefully it will become clear that although considerable progress has been made in both directions, our understanding of these multifunctional enzymatic systems and our ability to manipulate them productively is still in its infancy. In concluding, I speculate on future research directions that are likely to emerge from studies on modular PKSs.

II. Discovery of Modular Polyketide Synthases

Long before the discovery of modular PKSs themselves, a wealth of indirect information was gained regarding the biosynthetic properties of modular PKSs through incorporation experiments with [^{14}C]-, [^{13}C]-, [^{18}O]-, and [^2H]-labeled substrates and inter-

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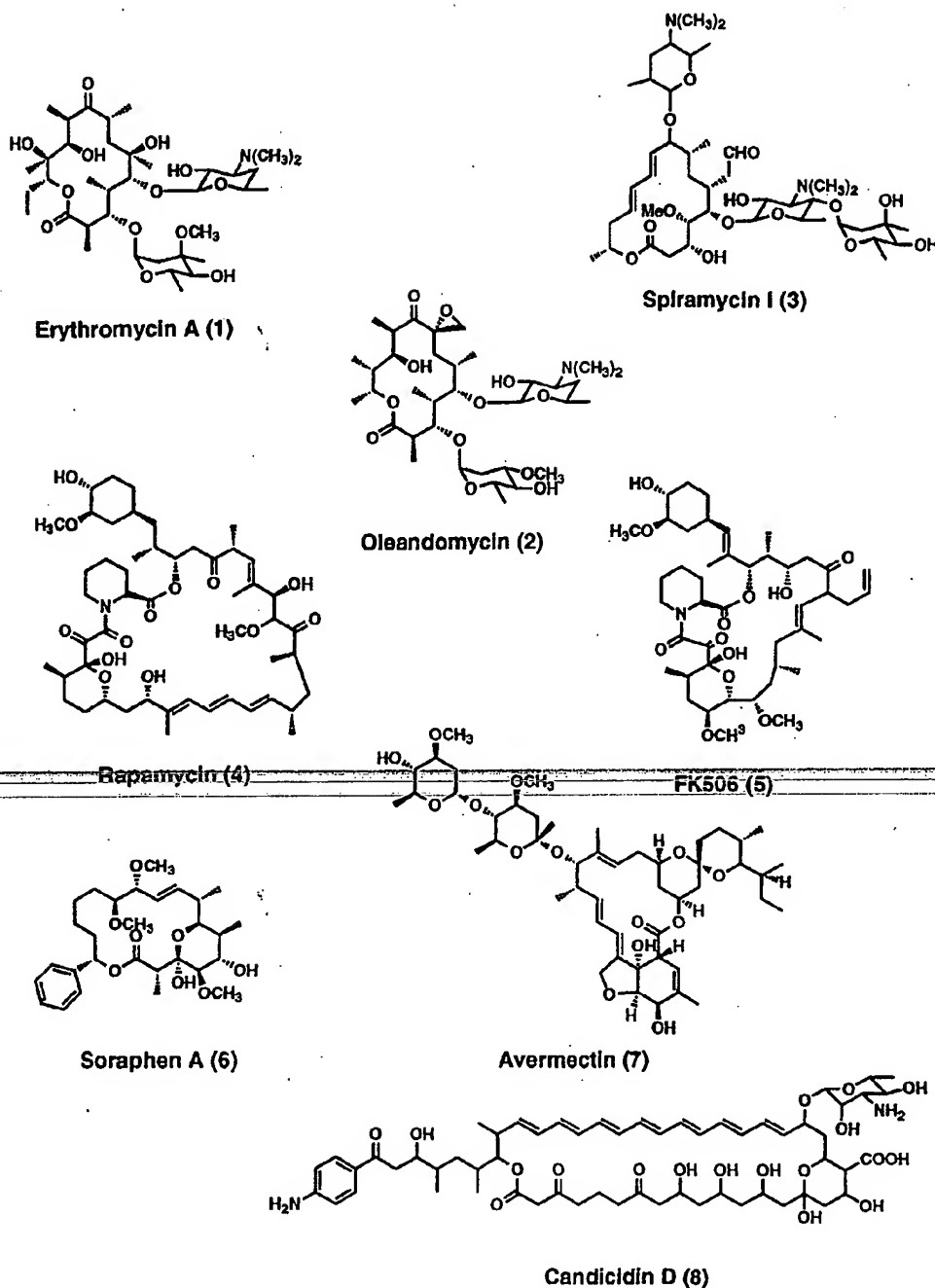


Figure 1. Structures of selected natural products derived from modular polyketide synthases.

mediate analogs (reviewed in an excellent monograph by O'Hagan¹). For example, isotope-labeling studies demonstrated that the carbon chain backbones of natural products such as erythromycin, tylosin, monensin, and avermectin are derived through C–C bond formation between acetate, propionate, and butyrate building block units (Figure 2a). More recently, the incorporation of exogenously added analogs of putative biosynthetic intermediates 9–11 into the corresponding polyketides 12 and 13 has unequivocally proven that modular PKSs act via a processive mechanism in which the oxidation level and stereochemistry of the growing polyketide chain is adjusted immediately after each step of polyketide chain elongation^{2,3} (Figure 2b). However, the biochemical basis for these highly controlled synthetic processes

remained virtually unknown until the advent of molecular genetic tools in this problem area.

In the 1980s, during the course of their genetic analysis of secondary metabolite biosynthesis in various *Streptomyces* species, Hopwood and his collaborators made the monumental discovery that the genes responsible for the biosynthesis, regulation, and self-resistance of bacterial natural products are clustered in the genomes of producer organisms (for review, see ref 4). On the basis of their work, a variety of genetic strategies have been developed to take advantage of nature's benevolence in order to clone complete biosynthetic gene clusters of interest. Frequently used strategies include complementation of blocked mutants, transfer of partial or complete pathways in a surrogate host, homology-based gene

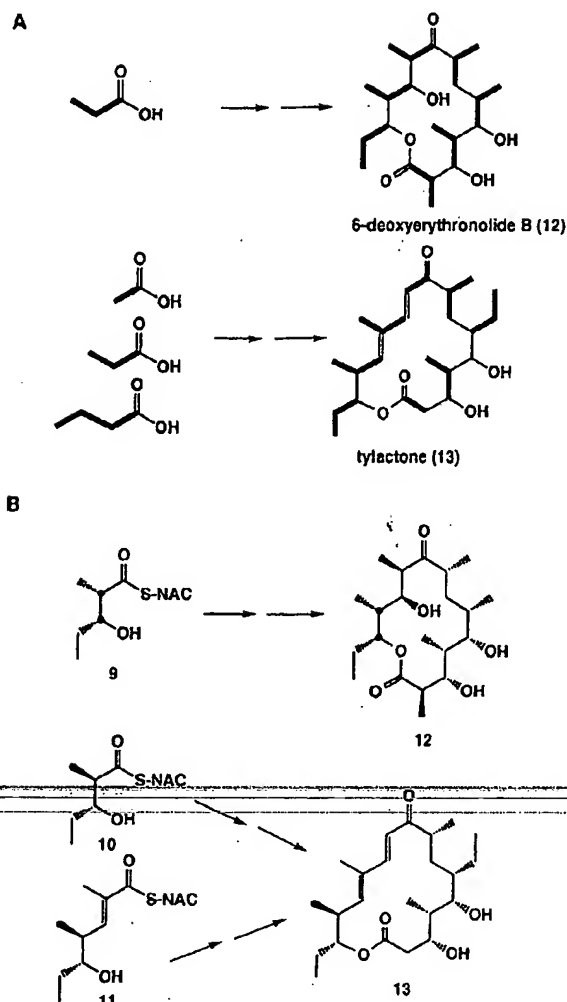


Figure 2. Conversion of (a) carboxylic acid building blocks and (b) advanced intermediates into the products of modular polyketide synthases.

isolation, identification of resistance gene(s) through selection in a heterologous host, and reverse genetics based on limited amino acid sequence of a purified pathway enzyme. In the process of these studies, a variety of cloning vectors, selectable markers, and gene probes have been added to the genetic toolbox

for studying secondary metabolism in the actinomycetes.

Building upon these discoveries and technological developments, the groups of Peter Leadlay at the University of Cambridge and Leonard Katz at the Abbott Laboratories independently cloned the erythromycin gene cluster.^{5,6} Whereas the Cambridge group accomplished this goal by chromosome-walking away from the *ermE* self-resistance gene, the Abbott group functionally cloned the PKS genes through complementation of mutants inactivated in the production of the polyketide aglycon. DNA sequence analysis revealed that the structural genes responsible for the formation of 6-deoxyerythronolide B (6-dEB; 12) consist of three contiguous open reading frames of ~10 kb each, encoding three large (~3000 amino acid) multidomain proteins, designated deoxyerythronolide B synthase (DEBS) 1, 2, and 3 (Figure 3). Furthermore, sequence comparisons also showed that each of these proteins consists of 8–10 domains with considerable sequence similarity to enzymes responsible for each of the individual steps of fatty acid biosynthesis. Moreover, these domains are arranged such that each protein contains two functional units or modules, each of which carries all the requisite catalytic activities for one of six cycles of polyketide chain elongation and reductive modification of the resultant β -ketoacyl thioester. Thus, although the enzymes involved in 6-dEB biosynthesis were clearly related to their counterparts in fatty acid synthases as well as other PKSs, this groundbreaking genetic analysis had revealed a fundamentally new paradigm for nontemplate biocatalysis, designated "modular PKSs".

III. Genes Encoding Modular Polyketide Synthases

Since the discovery of the DEBS genes, the involvement of modular PKSs in the biosynthesis of several other complex polyketides has been reported. Although some variations have been observed in the content and organization of different systems, the key features of the modular hypothesis remain unchanged.

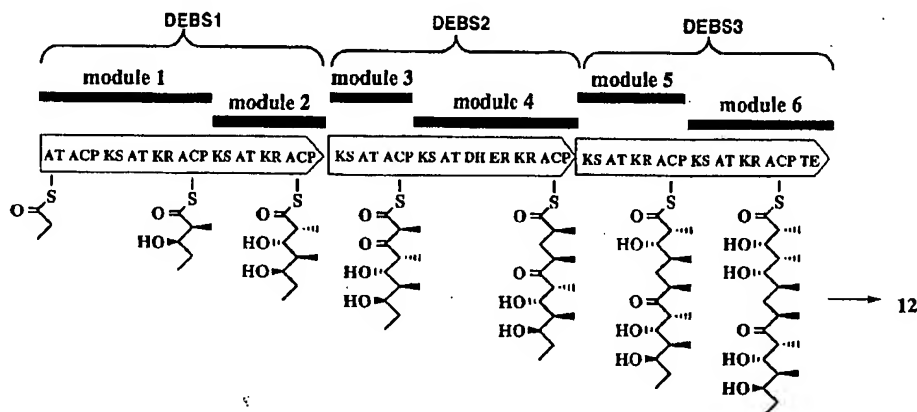


Figure 3. Genetic organization of the erythromycin polyketide synthase, DEBS, which catalyzes the biosynthesis of 6-dEB. The PKS consists of the polypeptides DEBS1, DEBS2, and DEBS3 (each MW > 300 kDa) that each possess two modules. Key: KS, ketosynthase; AT, acyltransferase; ACP, acyl carrier protein domain; KR, ketoreductase; DH, dehydratase; ER, enoylreductase; TE, thioesterase.

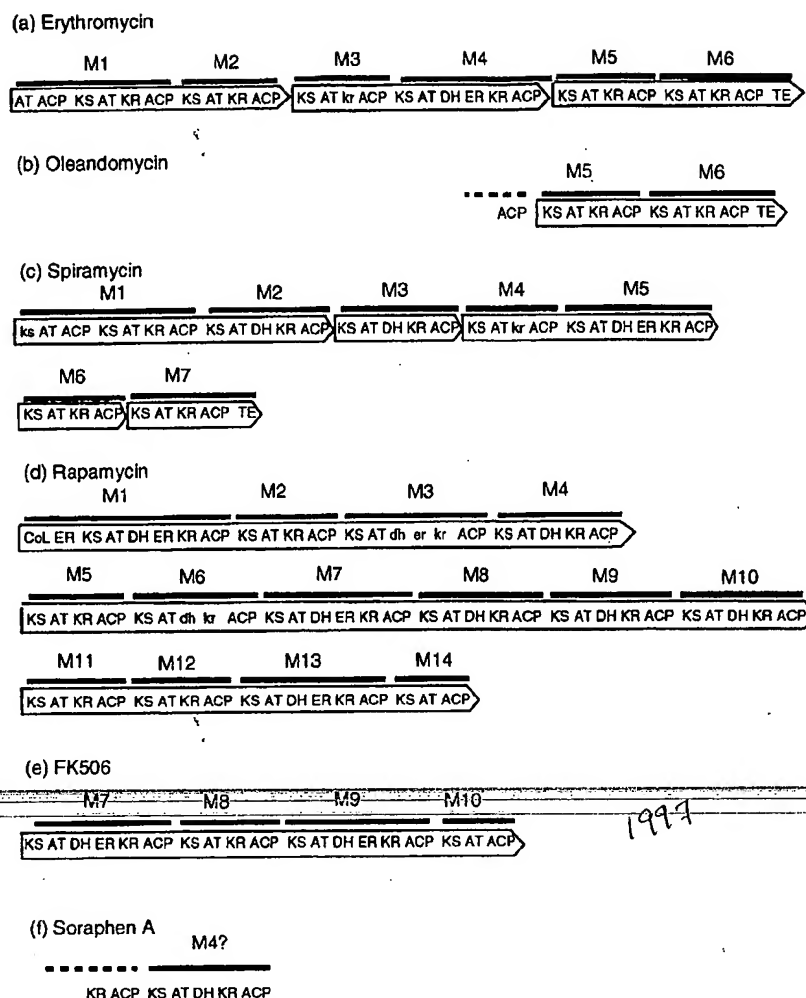


Figure 4. Domains of modular polyketide synthases that have been partially or completely characterized: (a) erythromycin, (b) oleandomycin, (c) spiramycin, (d) rapamycin, (e) FK506, and (f) soraphen A. Acronyms are as defined in the legend to Figure 3. Wherever identified, the PKS polypeptides are indicated as arrows. Domains that are believed to be functionally inactive are indicated in lower case. Uncertain module assignments are indicated with a question mark. For details, see text and references therein.

A. Erythromycin and Oleandomycin

Most of our knowledge about modular PKSs emanates from studies on the DEBS system. DNA sequence analysis of these genes led to the postulation of the now widely accepted model presented in Figure 3. Here, the acyltransferase (AT) domain at the N-terminus of DEBS1 initiates the polyketide chain-building process by transferring the propionyl-CoA primer unit, via the pantetheinyl residue of the first acyl carrier protein (ACP) domain, to the active-site cysteine of the ketosynthase of module 1 (KS1). The acyltransferase in module 1 (AT1) loads methylmalonyl-CoA onto the thiol terminus of the ACP domain of module 1. KS1 then catalyzes the first polyketide chain-elongation reaction by decarboxylative condensation between the methylmalonyl and propionyl residues, resulting in the formation of a 2-methyl-3-ketopentanoyl-ACP thioester. The latter intermediate is then reduced by the ketoreductase of module 1 (KR1), giving rise to enzyme-bound (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP. At this point, module 1 has finished its task and the diketide product is transferred to the core cysteine of KS2, whereupon it undergoes another round of condensation and reduction, resulting in the formation of the corresponding triketide. This process is repeated several times, with each module being responsible for a separate round of polyketide chain elongation and reduction, as appropriate, of the resulting β -ketoacyl thioester. Finally, the thioesterase (TE) at the C-terminus of DEBS3 is thought to catalyze release of the finished polyketide chain by lactonization of the product generated by module 6.

It should be noted that although the domain organization of DEBS is in complete agreement with the wealth of information available from isotope-labeling analysis of 6-dEB biosynthesis, unequivocal *a priori* deduction of product structure from gene sequence would not have been possible for three reasons. First, the stereochemical features of 6-dEB cannot be deduced from primary sequence information alone. Second, although module 3 of DEBS does not catalyze reduction of the β -carbonyl of the growing chain, it possesses a KR domain (albeit one whose amino acid sequence deviates significantly from that of the other KR domains around the nucleotide binding site; not shown in Figure 3 but shown in Figure 4). Finally, the regiospecificity of cyclization

is not overtly prescribed in the organization or sequence of DEBS domains. Ameliorating these limitations of sequence analysis of modular PKS genes represents a major challenge in the field.

In 1965, Celmer first noted the striking stereochemical similarities between the structures of the 12-, 14-, and 16-membered macrolide antibacterial agents.⁷ Given these similarities, it was not surprising that the genes encoding the erythromycin (Figure 4a) and oleandomycin (2) (Figure 4b) PKSs are remarkably similar, as illustrated by the cloning and sequencing of the bimodular gene responsible for the final two rounds of condensation in deoxyoleandolide biosynthesis.⁸

B. Spiramycin

The genes encoding the biosynthesis of the polyketide precursor of the 16-membered macrolide, spiramycin (3), have been cloned.⁹ Analogous to the erythromycin PKS, this modular PKS includes seven modules whose organization is colinear with the biosynthetic order (Figure 4c). However, as opposed to erythromycin, there are three unimodular open reading frames in the spiramycin PKS gene cluster. Furthermore, in addition to acyltransferase and acyl carrier protein domains, the loading domains also include a ketosynthase domain whose active-site cysteine is replaced with a glutamine (and therefore is presumably inactive). In addition to methylmalonyl transferase domains, the spiramycin PKS also includes malonyltransferase and ethylmalonyl transferase domains, and possibly a transferase for yet another CoA derivative related to malonyl-CoA but of unknown structure (which exists in the penultimate module).

C. Rapamycin and FK506

The entire gene cluster for rapamycin (4) biosynthesis has been cloned and sequenced from *S. hygroscopicus*, a rapamycin-producing organism.¹⁰ As predicted from the rapamycin structure, the PKS is comprised of 14 modules (Figure 4d). Noteworthy features of the rapamycin PKS include (a) noncolinearity of the PKS genes (the gene set shown in Figure 4d has been reorganized in a colinear order for convenience), (b) the existence of polypeptides containing four and six modules, (c) an unusual set of loading domains comprising a putative acyl-CoA ligase and an enoylreductase, possibly consistent with the occurrence of a substituted cyclohexanecarboxylic acid primer unit, and (d) an adjacent gene that encodes a pipecolate-incorporating enzyme, which presumably completes the rapamycin macrocycle and is homologous to modules from nonribosomal peptide synthetases.¹¹ Again, as in the erythromycin and spiramycin PKSs, unpredictable KR, DH, and/or ER domains were identified in two modules (shown in lower case in Figure 4), illustrating the difficulty of assigning intermediate and product structures based on gene sequences.

Both rapamycin and FK506 (5) bind to a family of proteins known as FK506-binding proteins (FKBPs). Protein-ligand interactions in both cases are very similar and are mediated through the portions of these polyketide natural products derived from the

last three extender units as well as the pipecolate and cyclohexanoyl residues. Not surprisingly, the last three modules of the two PKSs are very similar in amino acid sequence¹² (Figure 4e). The sequence of the remainder of the FK506 PKS has not yet been published.

Until the rapamycin PKS was sequenced, the database of modular PKS sequences only included sequences of extender AT domains with specificity toward methylmalonyl-CoA. The rapamycin PKS includes seven AT domains each with specificity toward malonyl-CoA or methylmalonyl-CoA. Comparative analysis of the AT domains from the rapamycin, erythromycin, and oleandomycin PKSs revealed that substrate specificity of AT domains could be unambiguously predicted from two short consensus sequences of 5–8 residues.¹³ At least one of these two motifs was found to be a good predictor of AT domain specificity in the case of the FK506 PKS modules.¹² Assuming that the predictive quality of these signature sequences remains good, this will be an important step toward the goal of deducing aspects of product structure from the sequences of modular PKS genes.

D. Soraphen

Soraphen (6) is a macrocyclic polyketide synthesized by the Gram-negative myxobacterium, *Sorangium cellulosum*. Recently, partial sequencing of the soraphen gene cluster has revealed the presence of a modular PKS-like open reading frame¹⁴ (Figure 4f). Although the amino acid sequence of only ~1.5 modules is available, it clearly illustrates that the modular PKS paradigm is broadly prevalent in bacteria, and is not merely confined to *Actinomyces* species.

E. Avermectin

The polyketide precursor of avermectin (7) has not been unambiguously identified as yet. Nevertheless, on the basis of available isotope-labeling results¹⁵ and the DEBS precedent, the avermectin PKS was expected to be comprised of 12 modules. Incomplete sequence analysis of the avermectin PKS has confirmed the existence of 12 modules.¹⁶

F. Candicidin/FR-008

The aglycon moieties of polyene macrolides such as candicidin (8) are large macrolactones derived from several condensation cycles. For example, the polyketide backbone of candicidin and a closely related product, FR-008, is presumably derived from 21 condensation cycles and cyclizes to form a 38-membered ring. Thus, if polyene macrolides are also synthesized by modular PKSs, these PKSs must be extremely large multifunctional enzyme complexes. Although the DNA sequence for no polyene PKS gene cluster has been reported to date, cloning, physical mapping, and Southern blot hybridization techniques have revealed that the FR-008 PKS is encoded over ~105 kb in the genome of *Streptomyces* sp. FR-008.¹⁷ Assuming ~5 kb for each PKS module, this is in striking agreement with the expectation for the 21-step condensation process required for the synthesis of the FR-008 carbon chain. Furthermore, consistent

with the presence of a *p*-aminobenzoic acid primer unit in the polyketide backbone, one end of the gene cluster appears to carry *p*-aminobenzoic acid synthase and ligase genes.

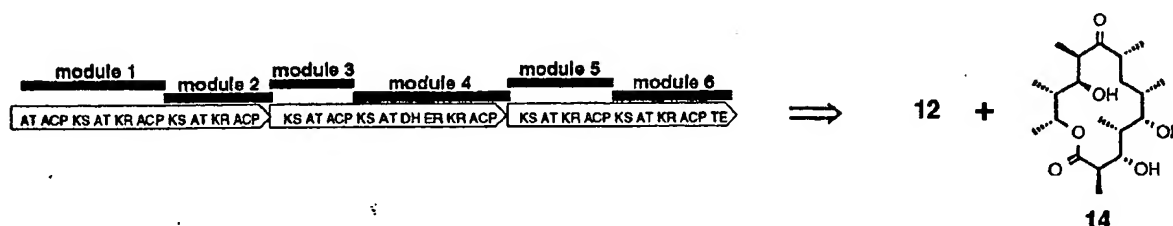
IV. Enzymology of the Erythromycin Polyketide Synthase

A. Expression Hosts, Vectors, and Model Systems

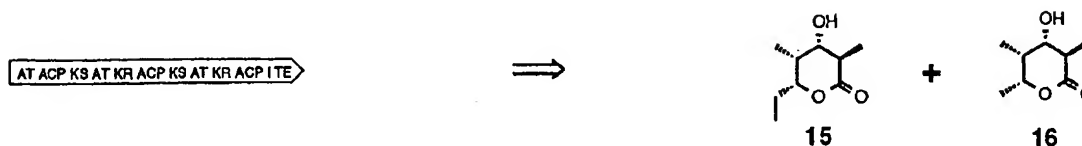
In addition to being the first modular PKS to be discovered, DEBS and its derivatives have proven to be excellent model systems for fundamental genetic and biochemical investigations into the properties of modular PKSs. Three different strategies have been explored for the construction and analysis of recombinant forms of DEBS. First, the chromosomal copies of the genes encoding DEBS in *S. erythraea* have themselves been targets of genetic engineering (for example, see ref 6). This approach takes advantage of the natural host, whose regulatory networks, PKS folding, post-translational modification, and assembly mechanisms, *in vivo* precursor pools, and export/resistance mechanisms have presumably been optimized over evolutionary time. However, it has the significant disadvantage that chromosomal genetic engineering in *S. erythraea* is at best a tedious and inefficient process. To circumvent these difficulties,

Escherichia coli using T7-promoter based systems (for example, see ref 18). Although DEBS proteins could be produced in *E. coli*, they were unable to catalyze synthesis of polyketides *in vivo* or *in vitro*. While the exact reasons for this inability remain unclear, the ACP domains were found to lack pantetheinylation, indicating at least one source of inactivity. In 1994, using a specially engineered host-vector system for the expression of recombinant polyketide synthases, we succeeded in expressing the complete set of DEBS structural genes in *Streptomyces coelicolor*, which does not produce any known modular PKS-derived natural product.¹⁹ The resultant strain produced substantial quantities (>40 mg/L) of 6-dEB (12), as well as a novel cometabolite, 8,8a-deoxyoleandolide (14) (>10 mg/L) (Figure 5a). The production of 6-dEB demonstrated that DEBS1, 2, and 3 carried all the necessary biosynthetic activities to support polyketide backbone formation and cyclization. Furthermore, it was evident that ancillary activities required to phosphopantetheinylate the ACP domains were present in the heterologous host and that the recombinant DEBS was fully functional. That 6-dEB is being formed by the normal biosynthetic pathway was confirmed by the incorporation of [^{13}C]propionate, giving rise to the expected labeling pattern in the ^{13}C NMR spectrum of the resultant sample of 6-dEB. In an analogous experiment, the starter unit of 8,8a-deoxyoleandolide was labeled by [$1,2\text{-}^{13}\text{C}_2$]acetate, thereby confirming that

(a)



(b)



(c)

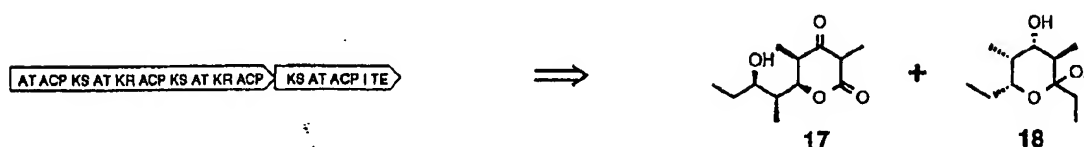


Figure 5. Selected model systems for modular polyketide synthases: (a) the full DEBS system; (b) a two-module derivative of DEBS, designated DEBS1+TE or DEBS1-TE; and (c) a three-module derivative of DEBS, designated DEBS1+module 3+TE.

DEBS can tolerate an acetate starter in place of its normal propionyl-CoA substrate. The utilization of acetyl-CoA as a starter is presumably due to the lower intracellular concentration of propionyl-CoA in *S. coelicolor* as compared to the native erythromycin producer, *S. erythraea*. Since then, this heterologous expression approach, which combines the power of *E. coli* molecular genetics with the well-known polyketide production capability of a streptomycete, has become the method of choice for the production of wild-type or engineered modular PKS proteins as well as their metabolic products.

Whereas the complete DEBS system offers a wealth of opportunities for genetics-led studies on modular PKSs, the sheer size of the DNA that encodes DEBS (> 30 kb) makes it a technically demanding target for genetic engineering. Current approaches to introduce mutations into the full DEBS system rely on the use of *in vivo* genetic engineering techniques (described in ref 19), which lack the tremendous versatility and facility of the standard (*in vitro*) protein-engineering toolbox. To overcome these technical limitations, two simplified forms of DEBS have been engineered. The first one, designated DEBS1+TE or DEBS1-TE, was independently developed by us²⁰ and others,²¹ and is comprised of the first two modules of DEBS (i.e., the DEBS1 protein) fused to the terminal TE domain of DEBS3 (Figure 5b). Introduction of this hybrid PKS gene into *S. coelicolor* resulted in production of substantially enhanced amounts of the expected triketide lactone (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ -lactone (**15**) (>20 mg/L). In addition, ~10 mg/L of the cometabolite originating from incorporation of an acetate starter, (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-hexanoic acid δ -lactone (**16**), was also produced. As with the formation of 8,8a-deoxyoleandolide from an acetate starter, the production of **16** most likely reflects some combination of the limited availability of propionyl-CoA in the *S. coelicolor* host strain; as well as the greater abundance of the alternative substrate acetyl-CoA. This conclusion is consistent with the observation that DEBS1+TE in *S. erythraea* exclusively produces **15**.^{21,22} Despite the relative simplicity of the system, DEBS1+TE harbors key molecular recognition features of the overall DEBS system: the first two modules generate methyl-branched carbon centers as well as secondary alcohols with both D and L stereochemistry. Furthermore, all protein domains required for catalytic activity reside in the same polypeptide, making it an attractive target for biochemical analysis. As will become clear below, it has become a workhorse model system for studies on modular PKSs.

More recently, a second simplified derivative of DEBS was described, whose size is sufficiently reduced so as to make it a target for manipulation by standard (*in vitro*) genetic engineering methodologies. This trimodular derivative, designated DEBS1+module 3+TE, includes intact DEBS1 together with module 3 of DEBS2 linked to the TE domain²³ (Figure 5c). When expressed in *S. coelicolor*, this mutant produced two novel tetraketide metabolites, CK13a (**17**), a six-membered ring lactone, and CK13b (**18**), a decarboxylated hemiketal,

in a 5:1 ratio. At least three properties of the trimodular PKS make it a potentially useful alternative to DEBS1+TE. First, the increased chain length and structural diversity of its polyketide products offers additional targets for intramodule manipulation without losing the benefit of cyclic and/or uncharged reporter products. Second, since module 3 is the smallest module of DEBS, module 3+TE is an attractive target for further biochemical and biophysical studies on a kinetically competent unimodular protein. Lastly, the chain-transfer event between module 2 and module 3 can readily be dissected *in vivo* and *in vitro* to understand the molecular basis for intermodule chain transfer within modular PKSs.

B. Cell-Free Activity

Until very recently, the detection of cell-free activity of modular PKSs has proven unsuccessful despite more than 30 years of intense efforts, presumably because of the difficulties in isolating fully active forms of these large multifunctional proteins from naturally occurring producer organisms, and because of the relative lability of intermediates formed during the course of polyketide biosynthesis. Fortunately, the situation appears to have changed with the availability of the well-behaved expression systems described above. In 1995 we demonstrated cell-free synthesis of 6-dEB by recombinant DEBS prepared from *S. coelicolor*.²⁴ The DEBS proteins were obtained at a level of ~3–5% total cellular protein and were found to catalyze the formation of 6-dEB (**12**), as well as the triketide lactone (**15**), upon addition of propionyl-CoA, (2*R*,*S*)-methylmalonyl CoA, and NADPH. A high phosphate concentration in the protein preparation and reaction buffers was found to be very important for the observed enzymatic activity, presumably by enhancing the assembly of the multienzyme complex via hydrophobic interactions. Polyketide synthesis was inhibited by both cerulenin and *N*-ethylmaleimide, both well-known inhibitors of the condensation reactions of fatty acid biosynthesis. The apparent k_{cat} parameters for the formation of 6-dEB and the triketide lactone were 0.5 and 0.23 min⁻¹, respectively,²⁵ pointing to the relative inefficiency of chain transfer from DEBS1 to DEBS2 in the *in vitro* system.

Analogous to the above studies with the complete DEBS system, similar (and more extensive) investigations have also been carried out by us and others on the formation of **15** by DEBS1+TE.^{24,26} This mini-PKS has been shown to be highly active as judged by a k_{cat} value of 4.8 min⁻¹²⁷ that is comparable to the estimated rate constant *in vivo* and by the fact that products can be synthesized on scales that facilitate structural analysis via NMR spectroscopy. The apparent K_m for methylmalonyl-CoA in DEBS1+TE-catalyzed synthesis of the tripropionate lactone is 24 μM .²⁵ In contrast, the K_m for propionyl-CoA is not easily measured, since the enzyme can readily decarboxylate methylmalonyl-CoA (or methylmalonyl-ACP) to generate a propionate primer which is turned over into **15** without any effect on the apparent k_{cat} .^{25,27}

Likewise, cell-free activity of the trimodular PKS, DEBS1+module 3+TE, has also been demonstrated.²⁷

As *in vivo*, the tetraketide lactone **17** is the dominant product in the presence of propionyl-CoA, (2*RS*)-methylmalonyl-CoA, and NADPH. The overall k_{cat} for tetraketide lactone synthesis is 0.23 min^{-1} , a value approximating that of the full DEBS system and considerably lower than that for DEBS1+TE. This suggests that either chain transfer between modules 2 and 3 is rate limiting in the biosynthesis of 6-dEB, or (more likely) that under currently used *in vitro* conditions the association of DEBS1 and module 3+TE is imperfect.

C. Protein Chemistry

Even before cell-free assays for the turnover of modular PKSs were developed, Leadlay and co-workers took advantage of the unusually large sizes of the three DEBS proteins to purify them from the natural erythromycin producer, *Saccharopolyspora erythraea*.²⁸ As predicted from the DNA sequence, DEBS1, DEBS2, and DEBS3 had relative molecular masses of 370, 380, and 330 kDa, respectively. DEBS3 was also purified from an *E. coli* expression system.¹⁸ Although *E. coli*-derived protein preparations were found to lack phosphopantetheinylation on the acyl carrier protein domains, the AT domains could successfully be acylated by their cognate substrates. More recently, using the overall activities of the multifunctional proteins as an assay through several purification steps, we reported the purification of DEBS1, DEBS1+TE, and module 3+TE to homogeneity.²⁷ In all of the above cases, gel filtration and equilibrium sedimentation analysis indicated that the individual proteins are associated as homodimers. A detailed map of proteolytic cleavage sites in DEBS1, DEBS2, and DEBS3 has been defined by Leadlay and co-workers through partial digestions with trypsin and elastase.²⁹ This map has served as a useful resource for studies on the orga-

nization and substrate specificity of certain domains within DEBS, as summarized below.

D. Module Assembly

Although the three-dimensional structure of a modular PKS remains unknown, the structures and arrangement of active sites in a PKS module are remarkably similar to those of vertebrate fatty acid synthases (FASs), which are also known to be dimeric. Moreover, two independent lines of investigation have recently established that analogous to the vertebrate FAS case, individual modules of a PKS dimer form head-to-tail homodimers. In one study Staunton *et al.* showed that an elastase fragment containing module 5 from DEBS cross-links as a dimer in the presence of dibromopropanone. A similar proteolytic fragment containing module 6 without ACP-6 fails to cross-link, suggesting that the ACP domain from one subunit is cross-linked to the KS domain of the other subunit.³⁰ Using a different approach, we obtained functional evidence for such head-to-tail association.³¹ In three derivatives of DEBS1+TE, the ketosynthase domain of module 1 (KS-1) or module 2 (KS-2), or the acyl carrier protein domain of module 2 (ACP-2) was inactivated via site-directed mutagenesis. As expected, the purified proteins were unable to catalyze polyketide synthesis. However, the KS-1/KS-2 and the KS-2/ACP-2 mutant pairs could efficiently complement each other and catalyze polyketide formation. In contrast, the KS-1 and ACP-2 mutants did not complement each other. Together these results support a structural model for modular PKSs in which the individual modules of a PKS dimer form head-to-tail homodimers, thereby generating two equivalent and independent clusters of active sites for polyketide biosynthesis (Figure 6). Each subunit contributes half of the KS and ACP domains in each cluster.

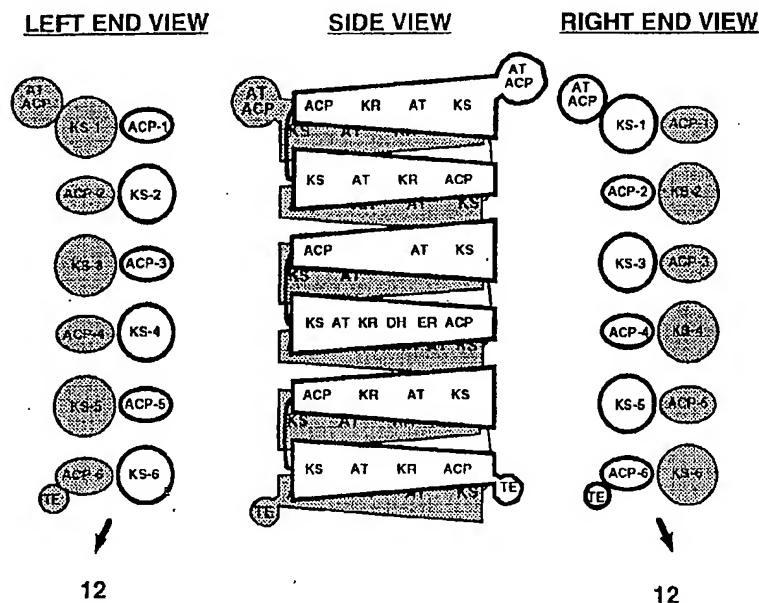


Figure 6. Proposed organization of KS and ACP domains in the dimeric erythromycin polyketide synthase. The two identical subunits are shaded and unshaded. (Side view) Each module forms a head-to-tail homodimer (= shaded + unshaded module), analogous to higher eucaryotic FASs. (End views) Two equivalent catalytic centers are present at opposite ends of the PKS complex. Each subunit contributes half of the KS and ACP domains to each catalytic center, and KS-*n* and ACP-*n* from opposite subunits interact during the synthesis of a polyketide molecule.

E. Molecular Recognition

Effective exploitation of the biosynthetic potential of modular PKSs crucially rests upon gaining a better understanding of the molecular recognition features of these multienzyme systems. Two levels of catalytic control are evident in modular PKSs. First, the modular structure of the proteins provides organizational control at the level of dictating the sequence of reactions to be employed in the overall catalytic cycle. Insights into how far this level of control can be manipulated are best derived from *in vivo* experiments involving deletion or insertion of modules or domains (see section V). Second, the intrinsic substrate specificities of some or all individual domains introduce an additional level of selectivity into the multistep transformation. As expected from the modular PKS paradigm, it has been shown that propionyl-CoA, which provides the primer unit for 6-dEB biosynthesis, specifically acylates an N-terminal proteolytic fragment of DEBS1, presumably at the active site serine of the loading AT domain.²⁹ Consistent with this observation, preincubation of DEBS1+TE with iodoacetamide fails to inhibit acylation by propionyl-CoA. Likewise, radiolabeled extender units derived from methylmalonyl-CoA are only found on proteolytic fragments containing the extender AT domains, but not the primer AT domain.²⁹ Surprisingly however, (2*S*)-methylmalonyl-CoA appears to be the exclusive substrate for polyketide chain elongation based on the stereospecific acylation experiments, suggesting that extender units that show a net retention of configuration (such as those incorporated by modules 1, 3, and 4 of DEBS) must undergo epimerization either prior to condensation or immediately after the condensation reaction.³²

Although DEBS shows absolute specificity for methylmalonyl extender units, it has a remarkably broad specificity toward alternative primer units. In addition to propionyl-CoA (or alternatively methylmalonyl-CoA, which undergoes enzyme-catalyzed decarboxylation to yield propionyl primers), both acetyl-CoA and butyryl-CoA can serve as surrogate chain initiators, giving rise to the corresponding triketide lactones **16** and **19**^{26,33} (Figure 7). Notwithstanding

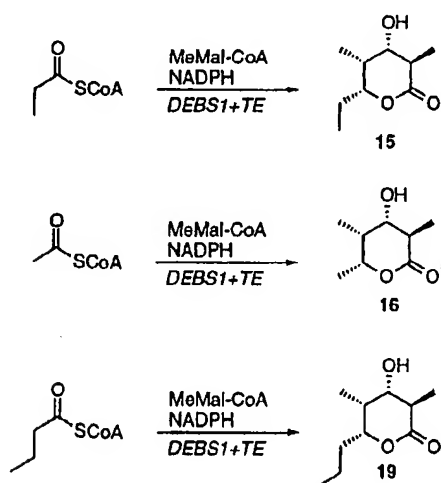


Figure 7. Relaxed primer unit specificity of the erythromycin polyketide synthase.

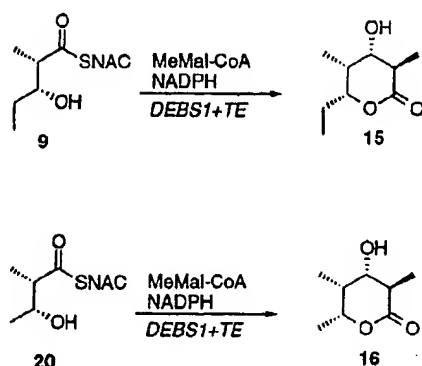


Figure 8. Diketide substrate specificity of module 2 of the erythromycin polyketide synthase.

this tolerance for different starter units, however, DEBS1+TE exhibits a 32-fold and an 8-fold kinetic preference toward propionyl primers over acetyl and butyryl primers, respectively, suggesting the existence of one or more active sites with discriminating molecular recognition features.²⁵

Guided by earlier results demonstrating that exogenously added *N*-acetylcysteamine (NAC) thioesters of chain-elongation intermediates could be incorporated into polyketide products by intact cells,^{2,3,34} we showed that in the presence of (2*RS*)-methylmalonyl-CoA and NADPH, DEBS1+TE could convert the diketide chain-elongation intermediate analog **9** to the triketide lactone **15** in a cell-free system²⁴ (Figure 8). More recently, it has also been confirmed that this diketide thioester is exclusively transacylated on to the active site cysteine of KS-2.³⁵ Analogs of **9** have been synthesized to probe the molecular recognition features of module 2 in DEBS. As expected from the above results, (2*S*,3*R*)-2-methyl-3-hydroxybutyryl-NAC thioester **20** is converted into **16** by DEBS1+TE³³ (Figure 8). Since butyryl-CoA is recognized as a primer, but **20** is recognized as a diketide, it is evident that the substitution pattern of an acyl chain, rather than its chain length, is the primary determinant of its ability to be specifically recognized by a given module. Furthermore, it also appears that the specificity toward acyl chains resides within KS domains. Further work along these lines should yield insights into the relative importance of the methyl-branched center vs the secondary alcohol in defining the diketide intermediate.

The relatively broad substrate specificity of DEBS toward unnatural substrates is also illustrated by excluding NADPH from various reaction mixtures. Incubation of DEBS1+TE with methylmalonyl-CoA and **9** in the absence of NADPH leads to formation of the keto lactone **21**, emphasizing the ability of the DEBS protein to mediate the formation of polyketides in a variety of oxidation states³⁶ (Figure 9). Furthermore, in the presence of propionyl-CoA and methylmalonyl-CoA alone, DEBS1+TE synthesizes the pyran-2-one **22**, presumably formed by lactonization of the unreduced diketoacylthioester product³³ (Figure 9). As will become clear from the discussion that follows, this tolerance of altered substrates by individual active sites in DEBS can be exploited for the engineered biosynthesis of novel polyketides by genetic as well as chemical strategies.

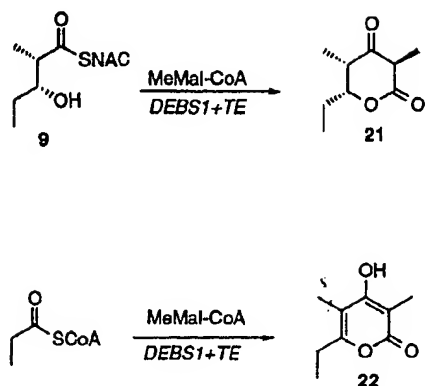


Figure 9. Tolerance of the erythromycin polyketide synthase toward partially reduced and unreduced substrates.

V. Biosynthesis of Novel Polyketides via Genetic Manipulation

In addition to facilitating fundamental investigations into the protein chemical properties of modular PKSs, molecular genetics has also been a powerful tool for the engineered biosynthesis of novel polyketides. In principle, domain mutagenesis of a modular PKS can be used to achieve loss of function, alteration of substrate specificity, or even gain of function. A rudimentary calculation might suggest that, even for a modest-sized modular PKS, the theoretical number of ways in which a functionally unique sequence of domains/modules can be constructed is astronomical. However, two potential barriers must be surmounted before the biosynthetic relevance of this genetic capability can be realized. First, strategies must be developed for engineering hybrid PKSs without deleteriously affecting the protein-protein interactions required for intramodule and intermodule chain processing. Second, the intrinsic tolerance of individual active sites toward alternative substrates must be decoded in order to design modules with functionally compatible domains and/or PKSs with functionally compatible modules. A growing number of studies along these directions are beginning to illustrate the actual scope as well as limitations of combinatorial biosynthesis.

A. Deletion of Modules

Initial evidence for the functional independence of upstream modules in a modular PKS from downstream ones came through heterologous expression of the DEBS1 gene by itself in *S. coelicolor*. The recombinant strain produced 1–3 mg/L of the triketide lactone **15**.³⁷ Although it was apparent that the

triketide can be released from DEBS1 alone without a requirement for a thioesterase (TE) domain, the low yield of **15** (relative to 6-dEB from DEBS using the same host-vector system) prompted us to explore the potential utility of the terminal TE domain from DEBS3 to accelerate product turnover from the first two modules. As described in section IV.A, the quantity of **15** produced by DEBS1+TE *in vivo* is comparable to that of 6-dEB produced by the full DEBS system, suggesting that turnover of DEBS1 is limited by chain release, and that the TE domain can effectively serve this purpose.²⁰ A similar result was obtained through an independent study involving transposition of the TE domain to construct an analogous fusion protein (designated DEBS1-TE by the authors) in the chromosomal DEBS genes of *S. erythraea*.²¹ Likewise, as described in section IV.A, the trimodular deletion mutant, DEBS1+module 3+TE, can efficiently generate products derived from the presumed tetraketide in the 6-dEB biosynthetic pathway.²³ Two shunt products are obtained from the tetraketide intermediate, the lactone **17** and the (decarboxylated) hemiketal **18**. Again, this is illustrative of the relatively broad substrate specificity of the TE for chain release, although none of the triketide or tetraketide reporter molecules provide insights into the cyclization specificity of the enzyme. To address this question, we constructed yet another deletion mutant of DEBS, containing DEBS1 and

DEBS2 plus a chimeric fifth module in which the ACP6-TE didomain region has now been fused just downstream of KR5. Assuming that KS5 and ACP6 could productively interact to catalyze a fifth condensation, it was expected that the mutant strain would be able to support five complete rounds of polyketide chain elongation and that the thioesterase/cyclase would catalyze release (and possibly macrolactonization) of the resultant polyketide. Indeed, the recombinant strain of *S. coelicolor* produced 20 mg/L of a completely new macrolactone, (8*R*,9*S*)-8,9-dihydro-8-methyl-9-hydroxy-10-deoxymethynolide (**23**) (Figure 10).²⁰ Since formation of this 12-membered macrolactone cannot be expected to occur spontaneously, the TE activity, which naturally supports the formation of a 14-membered ring product, presumably catalyzes exclusive formation of the 12-membered ring lactone, by esterification of the acyl thioester with the C-11 hydroxyl, in preference to the ordinarily kinetically favored generation of a δ -lactone by esterification with the C-5 hydroxyl. Together, these results demonstrate that upstream PKS modules are structurally and functionally independent from downstream ones and can therefore be used in isolation to produce novel biosynthetic products via genetic engineering.

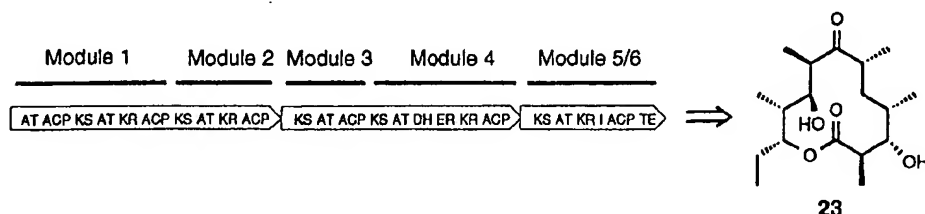


Figure 10. Engineered biosynthesis of a 12-membered macrolactone by a five-module derivative of the erythromycin polyketide synthase.

B. Loss of Function Mutagenesis within Modules

Two pioneering experiments demonstrating the biosynthetic versatility of modular PKSs involved the inactivation of individual domains within DEBS modules. Specifically, Katz and his co-workers generated a *S. erythraea* mutant carrying an ~80 amino acid in-frame deletion in the ketoreductase domain of DEBS module 5 (KR5) and demonstrated that this mutant produced **24**, a 6-dEB analog with a keto group at the predicted site, C-5 (Figure 11).⁶ This experiment provided not only direct experimental verification of the modular hypothesis suggested by the DEBS gene sequences, but established that the downstream domains in module 6 were capable of processing the modified polyketide chain-elongation intermediate generated by module 5. Similarly, Katz and co-workers also mutated the presumed NADPH binding motif of the enoyl reductase domain of module 4 (ER4). The resulting mutant strain produced a macrolide with the predicted $\Delta^{6,7}$ -anhydroerythronolide (**25**) skeleton (Figure 11).³⁸ More recently, a series of experiments leading to loss of function of the ketoreductase domain of module 2 (KR2) have been performed. Bedford et al. attempted to inactivate KR2 activity in DEBS1+TE using two different approaches.³⁹ In one case random PCR mutagenesis of the DNA encoding KR2 resulted in the isolation of a mutant protein with inactive KR2. In another approach the entire region in DEBS1+TE between the C-terminal end of AT2 and the N-terminal end of ACP2 was substituted with its homolog from module 3, which contains a KR-like domain with no apparent activity (Figure 11). Both recombinant PKSs produced the expected triketide ketolactone **21**, consistent with the observations of Katz and co-workers that inactivation of KR domains within modules does not affect the activity of the "core" domains (i.e., the KS, the AT, and the ACP).

Furthermore, the module 2/module 3 hybrid PKS also suggests that the relatively unconserved intervening region between AT and ACP domains of modules (~200 amino acid residues in the six modules of DEBS) does not crucially influence module assembly or activity. Together, these results suggest that inactivation of reducing domains can be readily accomplished in a variety of ways, and that in at least some cases the intermediates can be efficiently processed by downstream modules.

C. Change of Specificity Mutagenesis within Modules

In addition to domain and module inactivation, the modular PKS paradigm also offers potential opportunities to alter product structure by manipulating the specificity of individual enzyme-catalyzed reactions through domain replacement and possibly even site-directed mutagenesis. Two types of changes in this category of mutations can be envisioned: varying the choices of primer or extender units, and varying the stereochemistry at any chiral center. As discussed in section IV.E, acyltransferase domains are the primary determinants of primer and extender unit specificity in DEBS. Thus, replacement of an AT domain with one of different specificity would be expected to yield a modified polyketide, so long as the other activities within the engineered PKS are unaffected. The feasibility of altering primer unit specificity through interchange of loading AT domains was recently illustrated by Kuhstoss and co-workers, who replaced the loading AT-ACP of the spiramycin PKS (which uses an acetyl primer) with that of the tylosin PKS (which uses a propionyl primer).⁹ The resulting mutant (which was constructed in a genetic background that prevented post-PKS modifications) produced the expected hybrid polyketide aglycon **26** (Figure 12). Likewise, the

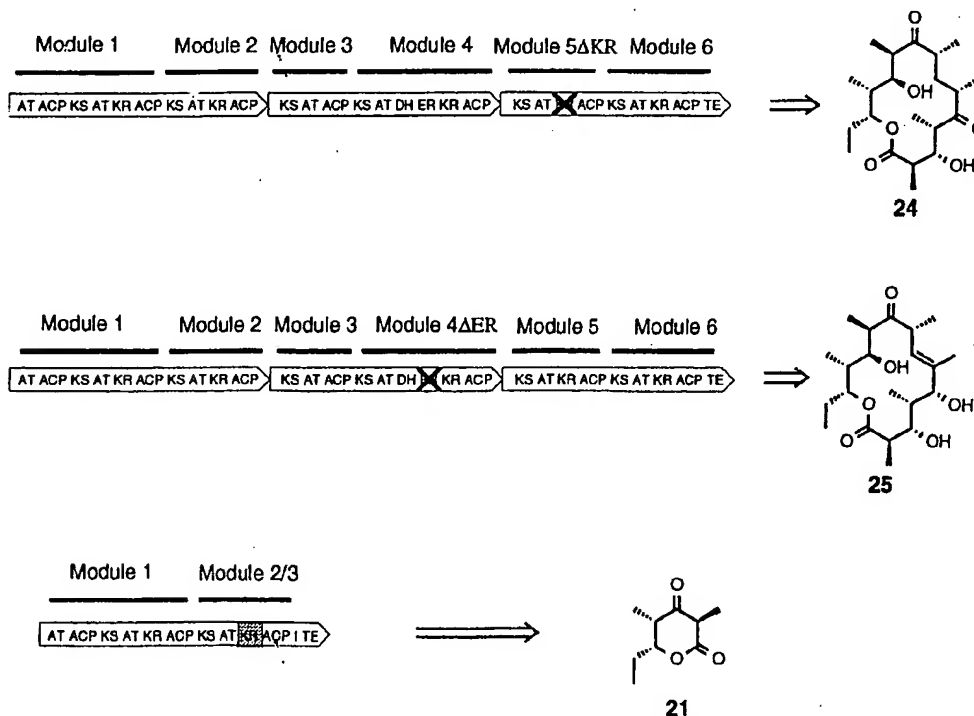


Figure 11. Loss-of-function mutagenesis of domains in modular polyketide synthases.

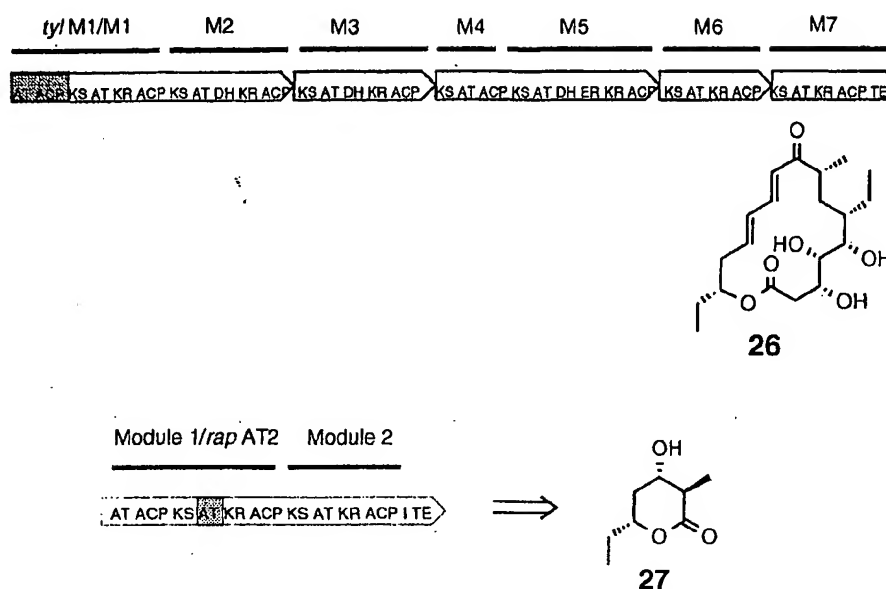


Figure 12. Alteration of substrate specificity of a modular polyketide synthase via domain substitution.

feasibility of altering extender unit specificity was demonstrated by Leadlay and co-workers, who replaced AT1 in DEBS1+TE, which introduces a propionate extender into the polyketide backbone, with AT2 of the rapamycin PKS, which has specificity for acetate extender units (Figure 12).⁴⁰ The resulting hybrid PKS synthesized the expected desmethyl triketide **27** when introduced into *S. coelicolor* CH999. Other than acetate and propionate extender units, macrolide synthases are also known to incorporate butyrate, glycolate, and possibly other extenders into their backbones. In addition to manipulating substrate specificity, in principle the stereospecificity of domains that generate chiral carbon centers could be altered. However, at the present time the enzymatic basis for stereochemical control in DEBS (or other modular PKSs) remains an enigma.

D. Gain of Function Mutagenesis within Modules

Much of the structural diversity within the polyketide superfamily of natural products is due to the ability of PKSs to vary the reduction level of every alternate carbon atom in the backbone. Thus, although the ability to introduce heterologous domains such as ketoreductases, dehydratases, and enoylreductases into modules that naturally lack these functions would be a powerful tool for generating structural diversity in unnatural polyketide libraries, it is particularly challenging due to the fact that these enzymatic activities are nonessential and must successfully compete for substrates with the downstream module. Recently, we demonstrated the feasibility of gain-of-function mutagenesis in a modular PKS. Specifically, KR2 in DEBS1+module 3+TE was

replaced with the didomain DH-KR from module 4 of the rapamycin PKS (Figure 13).⁴¹ Not only does this chimeric multifunctional enzyme catalyze β -ketoreduction and regiospecific dehydration of the covalently bound triketide intermediate, but the resulting product is transferred to and faithfully processed by module 3 of DEBS, leading to the production of decarboxylated tetraketide KOS009-7 (**28**). Interestingly, the chimeric module generated in the process is also significant from a medicinal context, since in the context of the full DEBS system it could considerably simplify access to ketolides, a newly discovered class of semisynthetic antibiotics with potent activity against a broad range of drug-sensitive and resistant bacterial pathogens.^{42,43}

VI. Precursor-Directed Biosynthesis of Novel Polyketides

Although nature's polyketide biosynthetic strategy is capable of yielding both diversity and complexity, it is fundamentally constrained by the repertoire of metabolically accessible building blocks, as well as by the available set of enzymatic activities that can be harnessed into a given module. Within these constraints, the tools of molecular genetics can be effectively applied to harness the biosynthetic potential of modular PKSs, as summarized in section V. However, the remarkable elasticity in the molecular recognition features of these multifunctional enzyme assemblies suggests that their catalytic utility could be considerably amplified if the above constraints could be relaxed. In theory this could be accomplished by using (nonbiological) synthetic substrates in the presence of cell-free PKS enzymes. In practice

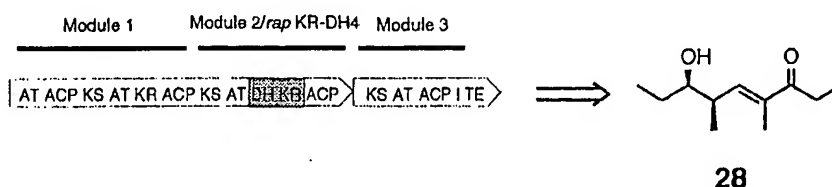


Figure 13. Gain-of-function mutagenesis of modular polyketide synthases by domain insertion.

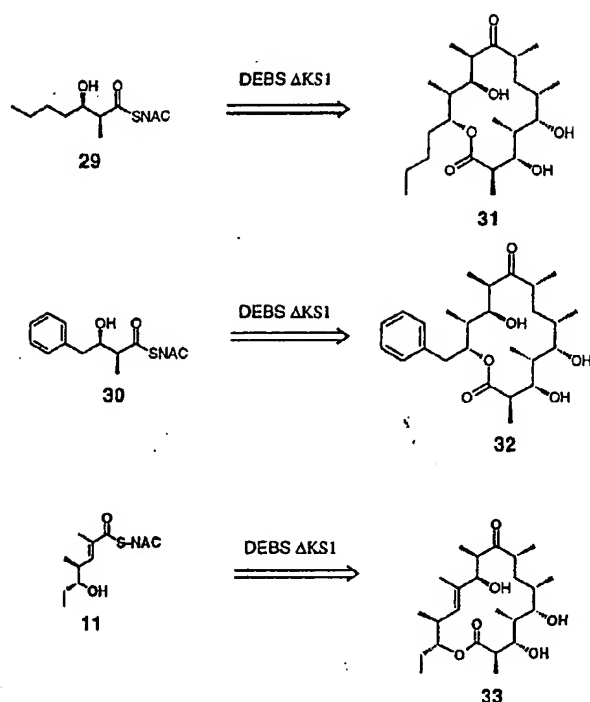


Figure 14. Precursor-directed biosynthesis of novel polyketides using genetically blocked modular polyketide synthases.

however, cell-free synthesis is limited by access to preparative quantities of enzymes (current practices limit the scale of cell-free polyketide synthesis to $\sim 1 \mu\text{mol}$) and by the absence of satisfactory regeneration systems for carboxylated CoA thioesters and NADPH. An alternative fermentation-based approach would combine the capacity of modular PKSs for the controlled generation of structurally complex products with the virtually unlimited repertoire of modern organic chemistry for the synthesis of substructures with diverse functionalities. The strategy has been recently illustrated by application to the erythromycin pathway.⁴⁴ Through site-specific mutagenesis, a genetic block was introduced in KS-1 of DEBS. Exogenous addition of diketide mimics **29**, **30**, and **11** to small-scale cultures of this null mutant resulted in highly selective multimilligram production of unnatural polyketides **31–33** (Figure 14). In particular, the unexpected incorporation of **11** (which is an epimer of the presumed triketide intermediate in the biosynthetic pathway leading to **28**) and the cyclization of its fully elongated product into a 16-membered lactone underscores the flexibility of the DEBS modules. Further processing of **31** and **32** (but not **33**) by post-PKS enzymes of the erythromycin pathway results in the generation of novel erythromycin D (**34**) analogs **35** and **36** with antibacterial activity (Figure 15).⁴¹ Not only does this mutasynthetic strategy complement the existing toolbox for combinatorial biosynthesis well, but its simplicity makes it relevant to numerous interesting natural products with minimal, and possibly even no, biosynthetic information at the genetic level. Thus, a genetic (via mutagenesis) or chemical (via selective inhibition) block can be introduced in an early PKS-catalyzed step within an organism that produces a relatively uncharacterized polyketide of biological

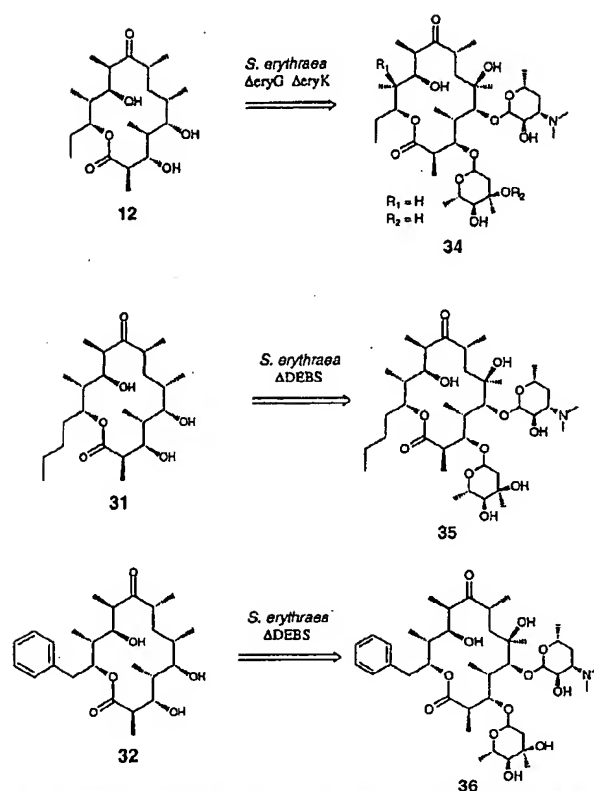


Figure 15. Bioconversion of structurally altered polyketide aglycons into novel erythromycin analogs.

and/or medicinal significance. The catalytic potential of this pathway could then be harnessed by feeding to the organism a cell-permeable synthetic molecule that is suitably designed so as to be selectively loaded on to any desired PKS module at the corresponding KS domain. Depending upon the intrinsic tolerance of the downstream pathway, the synthetic primer will be elaborated into a novel molecule with natural product-like complexity. A similar strategy has been developed in the context of the avermectin (**7**) pathway, where a mutant strain of *S. avermitilis* was identified through random mutagenesis that contains no functional 2-oxo acid dehydrogenase activity.^{45,46} Since the avermectin PKS is primed by isobutyric acid or isovaleric acid, which in turn is derived from branched chain amino acid degradation, the mutant was also unable to produce avermectin. However, when supplemented by isobutyric acid, isovaleric acid, or a variety of nonnatural carboxylic acids, the corresponding avermectins were isolated. The potential utility of such precursor-directed biosynthetic approaches is amplified in the context of the already broad range of genetic manipulations that can be performed on PKSs, as described in section V.

VII. Future Directions

The structural complexity and biological activities of polyketide natural products have been a source of inspiration to chemists and biologists alike. However, until recently, one's ability to alter the structures of these remarkable biomolecules in practical ways has been severely limited. The discovery of modular PKSs, and the ensuing technological and

conceptual breakthroughs that facilitate their exploitation, have opened up new horizons in the selective synthesis of complex organic molecules. It is becoming increasingly clear that nature has evolved two analogous but comparably powerful strategies for modular biosynthesis of complex biomolecules—a template-based approach (illustrated by the cellular machineries for DNA replication, RNA transcription, and polypeptide translation) in which a covalent template molecule is modularized, and a nontemplate-based approach (illustrated by PKSs) in which the catalyst itself is modularized. While our understanding of the mechanistic basis for nontemplate biocatalysis is still in its infancy, there can be little doubt that future studies on modular PKSs will throw light on exciting new concepts in biomolecular science and engineering.

VIII. Acknowledgments

This paper is dedicated to Professor Sir David Hopwood (John Innes Centre, U.K.) for his remarkable foresight and sustained efforts in bringing the concepts and tools of genetics to bear on the field of natural products chemistry. I would also like to acknowledge Dr. Leonard Katz (Abbott Laboratories) for introducing me to modular polyketide synthases, and Professor David Cane (Brown University) for an exciting and productive ongoing collaboration between our laboratories on the subject. I also wish to thank the students and postdoctoral fellows cited in references to publications from this laboratory for their exceptional creativity and dedication. Our studies on modular polyketide synthases have been supported in part by the National Institutes of Health (CA 66736), by an NSF Young Investigator Award, by a David and Lucile Packard Fellowship for Science and Engineering, and by gifts from Merck Research Laboratories, Schering Plough Research Laboratories, and Sankyo Co.

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